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(54) **ARABINOXYLO-OLIGOSACCHARIDES
USEFUL AGAINST GASTROINTESTINAL
INFECTIONS**

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CPC **A61K 31/716** (2013.01); **A23L 1/1016** (2013.01); **A23L 1/30** (2013.01); **A23L 1/308** (2013.01); **A61K 31/00** (2013.01); **A61K 31/715** (2013.01)

(58) **Field of Classification Search**

None
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to the use of oligosaccharides derived from arabinoxylan for use in the prevention and treatment of gastrointestinal infection. More particularly the invention provides a method for preventing or reducing the gastrointestinal infection of a animal or human being with bacteria associated with gastroenteritis through the supplementation of their diets with the said oligosaccharides.

14 Claims, 3 Drawing Sheets

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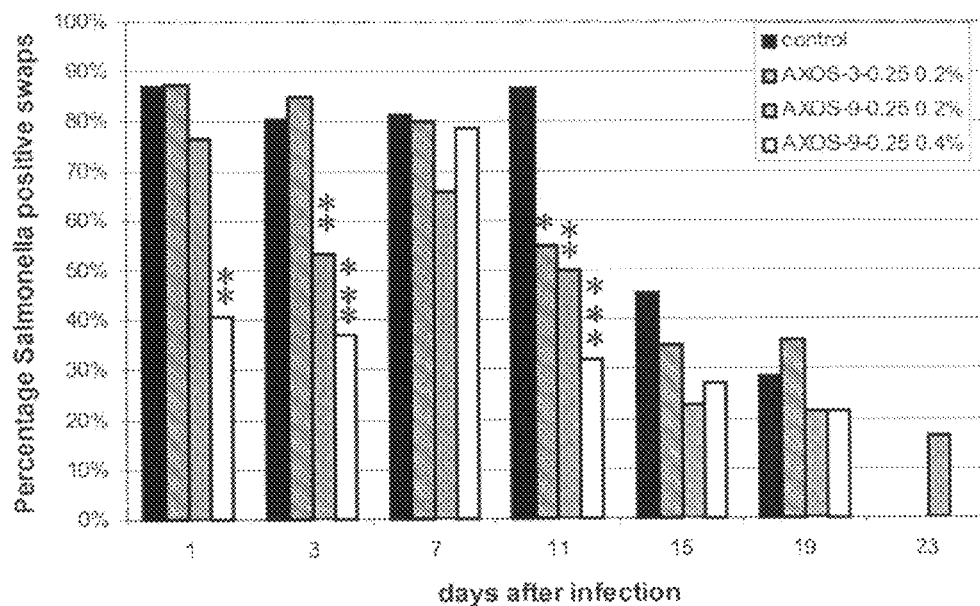


Figure 1

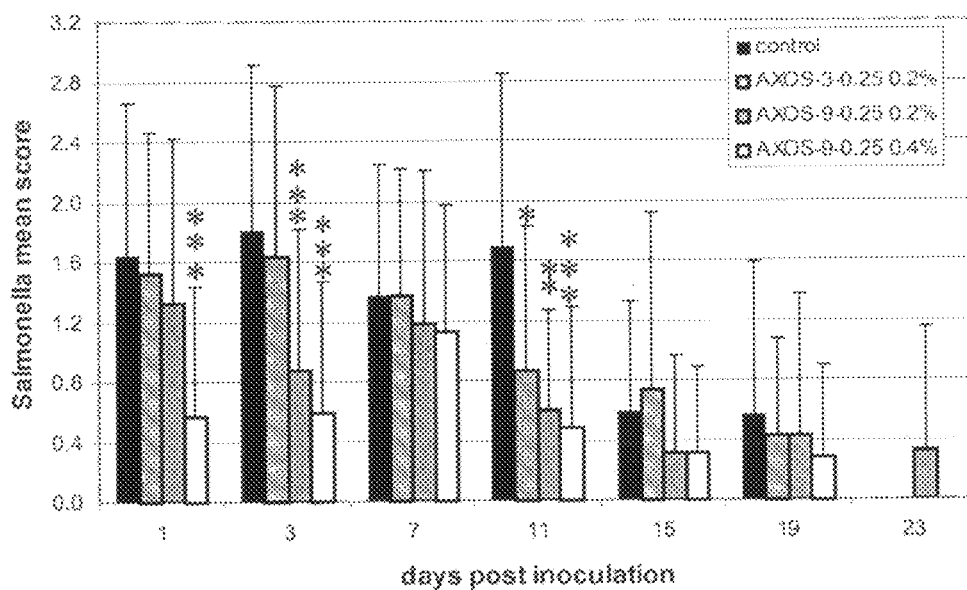


Figure 2

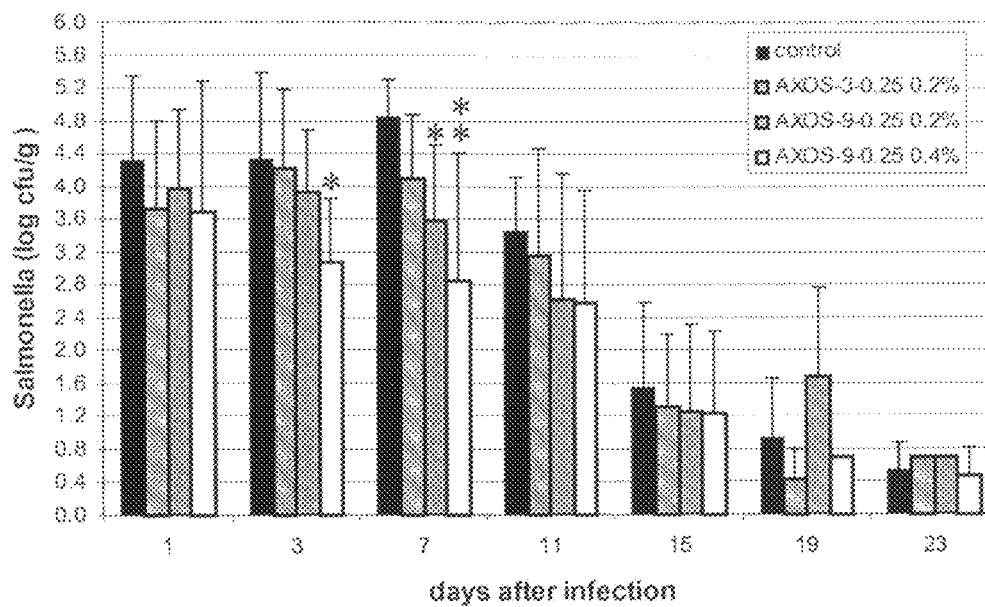


Figure 3

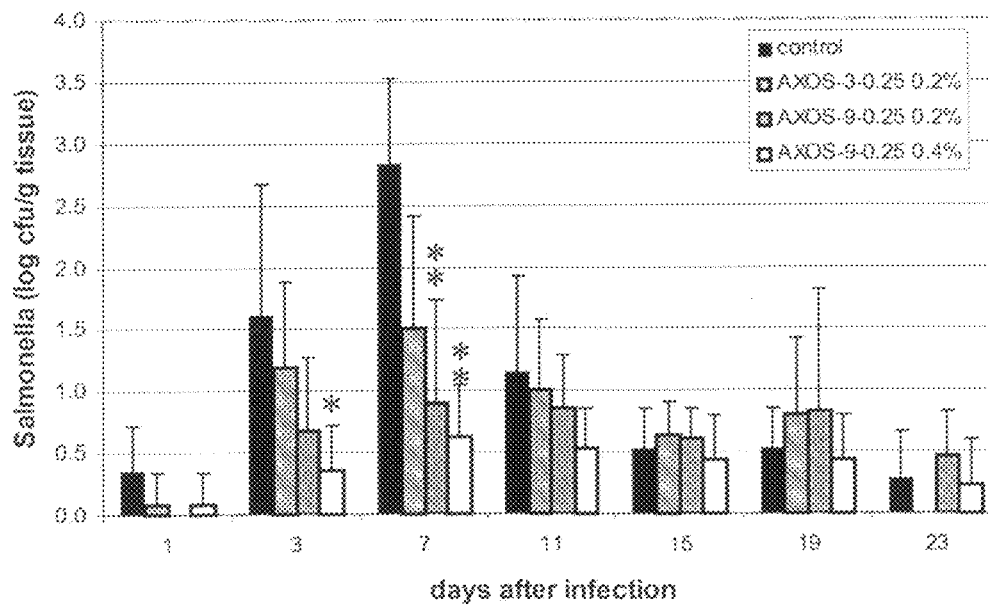


Figure 4



Figure 5

ARABINOXYLO-OLIGOSACCHARIDES USEFUL AGAINST GASTROINTESTINAL INFECTIONS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is the U.S. national stage of International Application No. PCT/EP2008/063044, filed Sep. 29, 2008, which claims the benefit of UK Patent Application No. GB0718974.9, filed Sep. 28, 2007.

FIELD OF THE INVENTION

The present invention relates to the use of oligosaccharides derived from specific arabinoxylans in the treatment and/or prevention of gastrointestinal infections in animals and human beings. More particularly the invention relates to a method for preventing or reducing the gastrointestinal infection of an animal or a human being with bacteria associated with gastroenteritis by supplementing their diets with the said oligosaccharides.

BACKGROUND OF THE INVENTION

Gastroenteritis is a significant problem in animal husbandry as well as in human medicine. Gastroenteritis is primarily caused by food-borne pathogens, such as bacterial species belonging to the *Salmonella*, *Campylobacter*, *Escherichia*, *Listeria*, *Clostridium*, or *Shigella* gen, which typically ingress through the lower parts of the gastrointestinal tract. In animal husbandry, gastrointestinal infections can be relatively well controlled by inclusion of sub-therapeutic doses of antibiotics in the feed. However, the preventative use of antibiotics is either banned or strongly discouraged in many countries due to the growing emergence of antibiotic-resistant pathogens. Likewise, antibiotics in human medicine are nowadays subject to more strict use policies. Alternative treatments include the use of dietary supplements such as short or medium chain organic acids, manno-oligosaccharides which interfere with mucosal attachment of pathogens, or fructan-based oligosaccharides which stimulate beneficial gut microbiota. Inoculation of animals with competitive-exclusion microbiota is another form of treatment.

There is a need to extend the range of preventive and therapeutic treatment methods against gastrointestinal infections, especially since some of the proposed control agents do not yield consistent results. For instance inulin and oligofructose have been reported to decrease the incidence of systemic infection by *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* in mice, whereas in rats it increased the levels of *S. enterica* ser. Enteritidis in the gut and promoted systemic translocation. Likewise, inclusion of a oligofructose from Jerusalem artichokes in chicken diet promoted colonisation of chicken caeca by *Salmonella enterica* ser. Typhimurium, whereas oligofructose from chicory roots had the opposite effect.

Arabinoxylans are polysaccharides that occur in the cell wall of most monocotyledonous plant species, and are therefore abundantly present in cereal-based diets. They comprise a main chain of β -1,4-linked D-xylopyranosyl units to which O-2 and/or O-3 \square -L-arabino-furanosyl units are linked, together with minor substituents such as α -(methyl)glucuronyl, acetyl, feruoyl, and/or p-coumaroyl groups. Arabinooligosaccharides (hereinafter referred as AXOS), i.e. oligosaccharides derived by partial hydrolysis of arabinoxylan, have been demonstrated to stimulate the growth and improve

the feed intake when added to the diet of animals (WO03/015533), whereas uncleaved arabinoxylan is well known to hamper efficient food utilisation. AXOS has also been shown to stimulate beneficial microflora in the caeco-colonic compartment of the gastrointestinal tract (WO06/002495), while arabinoxylan promotes fermentation in the ileum. The effect of dietary supplement with arabinoxylan on the attachment *S. enterica* ser. Enteritidis to the ileum of broiler chicks has been investigated. It was found that dietary addition of 0.5% arabinoxylan from corn hull (average molecular weight 500,000) slightly promoted *Salmonella* attachment when the chicks were raised at 23° C., whereas a slight decrease in attachment was observed in chicks raised at the suboptimal temperature of 29° C., although none of the effects were significant.

SUMMARY OF THE INVENTION

In a first object the present invention provides a method for preventing or reducing the infection of an animal or a human being with bacteria associated with the occurrence of gastroenteritis, comprising enterally administering arabinoxyloligosaccharides having an average degree of polymerization between 3 and 50 and an average degree of substitution between 0.15 and 0.35. The present invention further provides the use of such arabinoxyloligosaccharides in the production of a human or veterinary medicine for preventing or reducing the infection of a subject with bacteria associated with the occurrence of gastroenteritis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effects of the addition to chicken feed of 0.2% by weight AXOS-3-0.25, 0.2% by weight AXOS-9-0.25 or 0.4% by weight AXOS-9-0.25 on the percentage of *Salmonella* positive cloacal swabs at different time points after inoculation with *S. enterica* ser. Enteritidis, as measured after direct plating of the swabs. For a given time point the values marked with a symbol above the bars are significantly different from the control group according to the Cochran-Q test (*: P<0.05; **: P<0.01; ***: P<0.001).

FIG. 2 shows the effects of the addition to chicken feed of 0.2% by weight AXOS-3-0.25, 0.2% by weight AXOS-9-0.25 or 0.4% by weight AXOS-9-0.25 on the *Salmonella* score of cloacal swabs at different time points after inoculation with *S. enterica* ser. Enteritidis. A score of 0 was given for no colonies, 1 for 1 to 10 colonies, 2 for 11 to 100 colonies, 3 for 101 to 1000 colonies, or 4 for more than 1000 colonies of *S. enterica*. Bars represent means of the measurements and error bars indicate the standard deviation. For a given time point the values marked with a symbol above the bars are significantly different from the control group according to the Kruskal-Wallis test (*: P<0.05; **: P<0.01; ***: P<0.001).

FIG. 3 shows the effects of the addition to chicken feed of 0.2% by weight AXOS-3-0.25, 0.2% by weight AXOS-9-0.25 or 0.4% by weight AXOS-9-0.25 on the occurrence of *Salmonella* in the caecal content at different time points after inoculation with *S. enterica* ser. Enteritidis. Bars represent mean log cfu per g caecal content, and error bars indicate the standard deviation. For a given time point the values marked with a symbol above the bars are significantly different from the control group according to the Kruskal-Wallis test (*: P<0.05; **: P<0.01; ***: P<0.001).

FIG. 4 shows the effects of the addition to chicken feed of 0.2% by weight AXOS-3-0.25, 0.2% by weight AXOS-9-0.25 or 0.4% by weight AXOS-9-0.25 on the occurrence of *Salmonella* in the spleen at different time points after inocu-

lation with *S. enterica* ser. Enteritidis. Bars represent mean log cfu per g spleen tissue, and error bars indicate the standard deviation. For a given time point the values marked with a symbol above the bars are significantly different from the control group according to the Kruskal-Wallis test (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

FIG. 5 schematically shows an overview of the study design of a clinical trial evaluating the effect of the daily intake of different dosages of arabinoxylo-oligosaccharides (AXOS) on gastro-intestinal complaints in healthy human volunteers (WO=wash out).

DETAILED DESCRIPTION OF THE INVENTION

The pathogenic sequence of a bacterial gastrointestinal infection starts with the survival and growth of a pathogen in the lumen of the gastrointestinal tract. Therefore, the strengthening of a subject's colonization resistance against pathogens is an important strategy in the prevention of gastrointestinal infections. Moreover, an improved colonization resistance not only decreases the occurrence of infections but also reduces the severity of an incurred infection. The present invention shows that the oral administration of an appropriate amount of certain arabinoxylo-oligosaccharides significantly improves the gastrointestinal colonization resistance of a subject against pathogenic bacteria. Indeed, supplementing such arabinoxylo-oligosaccharides to the feed of a model animal orally infected with a gastrointestinal pathogen resulted in a clearly lower fecal excretion of that model pathogen in the faeces in the period following the infection. Moreover, the oral administration of such arabinoxylo-oligosaccharides reduced the translocation of the pathogen towards internal organs. Therefore, in a first object the present invention provides a method to reduce or prevent the infection of a human being or an animal with gastro-intestinal pathogenic bacteria. Said method comprises enterally administering a therapeutically effective amount of specific arabinoxylo-oligosaccharides to said human being or animal prior to or during the occurrence of an infection. Amongst others, the method of the present invention can be used to reduce or prevent the infection by gastro-intestinal pathogenic bacteria belonging to the *Salmonella*, *Campylobacter*, *Escherichia*, *Listeria*, *Clostridium*, *cholera*, *Vibrio*, *Yersinia*, *Aeromonas*, *Pleisomonas* or *Shigella* geni.

As used herein "arabinoxylo-oligosaccharides" refers to oligosaccharides derived from arabinoxylans comprising a main chain of β -1,4-linked D-xylopyranosyl units to which O-2 and/or O-3 α -L-arabino-furanosyl units are preferably linked. For the purpose of the present invention it is preferred that the average degree of substitution of the arabinoxylo-oligosaccharides varies between 0.15 and 0.35. Preferably, the average degree of polymerisation of the arabinoxylo-oligosaccharides used in the present invention is at least 3, more preferably at least 4, for instance at least 5. Preferably, the average degree of polymerisation of the arabinoxylo-oligosaccharides used in the present invention is at most 50, more preferably at most 20, most preferably at most 10. The average degree of substitution and the average degree of polymerisation can be determined by any method known in the art, e.g. as previously described by K. Swennen et al. in *J. Sci. Food Agric.* (2006) 86:722-731. In short, the carbohydrates in an arabinoxylo-oligosaccharides-containing preparation were hydrolysed using trifluoric acid and the resulting monosaccharides were reduced and converted to alditol acetates, which were then analyzed using gas chromatography. The total arabinoxylan-content was calculated as being 0.88 times the sum of the monosaccharides xylose and ara-

binose, while the average degree of substitution was determined as the arabinose-to-xylose ratio. The average degree of polymerization was calculated as the sum of the total xylose and arabinose divided by the amount of reducing end xylose.

Arabinoxylo-oligosaccharides suitable for use in the method according to the present invention can be obtained by partial hydrolysis of arabinoxylans extracted from cereals or cereal derived material. More preferably, these arabinoxylo-oligosaccharides may be obtained by hydrolysis of arabinoxylans extracted from bran, for instance wheat or rye bran.

When using the method according to the present invention for the prevention or treatment of the infection of an animal, e.g. a mammal, with gastro-intestinal pathogenic bacteria it is preferred to administer a therapeutically effective amount of the specific arabinoxylo-oligosaccharides through (together with) the feed. Preferably the animal feed is supplemented with at least 1 g, for instance at least 2 g, of arabinoxylo-oligosaccharides per kg feed. Preferably the animal feed is supplemented with at most 50 g, more preferably at most 10 g, most preferably at most 5 g, for instance at most 4 g of arabinoxylo-oligosaccharides per kg feed. The method of the present invention is useful as an element in the prophylaxis of livestock animals, poultry and pets. It more particularly contributes to the prophylactic management of the breeding of calves, chickens, turkeys, pigs and horses amongst others.

When using the method of the present invention in pet animals, such as cats and dogs, it may be advantageous to incorporate the daily effective amount of arabinoxylo-oligosaccharides in a daily serving of a pet food. Preferably, such pet food comprises at least 0.25 g, more preferably at least 0.5 g of arabinoxylo-oligosaccharides per daily serving of said pet food. Preferably, such pet food comprises at most 10 g, more preferably at most 5 g, most preferably at most 2.5 g of arabinoxylo-oligosaccharides per daily serving of said pet food. In a particular embodiment, it is preferred to include between 0.25 and 2 g, more preferably between 0.25 and 1 g of arabinoxylo-oligosaccharides per daily serving of a domestic cat food. In another particular embodiment, between 0.25 and 3 g, more preferably between 0.5 and 2 g of arabinoxylo-oligosaccharides are included per daily serving of food for dogs with a body weight below 10 kg, while between 0.25 and 5 g, more preferably between 0.5 and 3 g of arabinoxylo-oligosaccharides are included per daily serving of food for dogs with a body weight above 10 kg.

When using the method of the present invention in pet animals, such as cats and dogs, the daily effective amount of arabinoxylo-oligosaccharides can alternatively be included in a treat food for pets, such as biscuits or cookies. Typically, such pet treat food comprises between 0.25 and 5 g, more preferably between 0.25 and 2.5 g, for instance between 0.25 and 1 g of arabinoxylo-oligosaccharides per serving of said treat food.

When using the method according to the present invention for the prevention or treatment of the infection of a human with gastro-intestinal pathogenic bacteria, the arabinoxylo-oligosaccharides can be administered in admixture with a drink (e.g. water, milk, soft drink or beer), or in the form of a tablet, dietary supplement, candy, chewing gum or a food product. For the treatment of infants the arabinoxylo-oligosaccharides can be incorporated into an infant formula. The present invention can also be accomplished by incorporating the specific arabinoxylo-oligosaccharides in yoghurts, dairy products, fruit juice and cereal based products, such as bread, breakfast cereals, cookies and crackers amongst others. One aspect of the present invention relates to the finding that the consumption of between 0.25 and 10 g, preferably between 1 and 5 g, for instance between 1 and 3 g of arabinoxylo-

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oligosaccharides per day per human significantly reduces the presence in feces of several bacteria species associated with gastroenteritis. The reduced presence in the feces reflects a decrease of the presence of these bacteria in the intestinal flora, reducing the occurrence and severity of gastro-intestinal infections.

In a second object, the present invention relates to the use of said arabinoxylo-oligosaccharides in the production of a veterinary medicine for preventing or reducing the infection of an animal, e.g. a mammal, by gastro-intestinal pathogenic bacteria. Preferably said veterinary medicine can be incorporated into the feed of an animal in need thereof at a dose corresponding to between 1 and 50 g, more preferably between 1 and 10 g, most preferably between 1 and 5 g, for instance between 2 and 4 g of said arabinoxylo-oligosaccharides per kg feed. Preferably such veterinary medicine comprises at least 8% (w/w), more preferably at least 20% (w/w), most preferably at least 40% (w/w), for instance more than 60% by weight or more than 80% (w/w) of said arabinoxylo-oligosaccharides. Alternatively, said veterinary medicine may be added to the drinking water of the animal, preferably the veterinary medicine is added to water in order to obtain a weight concentration of said arabinoxylo-oligosaccharides between 0.25 and 20% (w/w), more preferably between 0.25 and 10% (w/w), most preferably between 0.25 and 5%. Furthermore, the said veterinary medicine may also be included into pet foods or in treat food for pets in order to supplement such pet foods with said arabinoxylo-oligosaccharides as indicated above.

In a third object the present invention relates to the use of said arabinoxylo-oligosaccharides in the production of a pharmaceutical composition for preventing or reducing the infection of a human being with gastro-intestinal pathogenic bacteria. The pharmaceutical composition of said medicament allows for the administration of a daily dose of between 0.25 and 10 g, preferably between 1 and 5 g, for instance between 1 and 3 g of arabinoxylo-oligosaccharides. Preferably said medicament comprises at least 8% (w/w), more preferably at least 20% (w/w), most preferably at least 40% (w/w), for instance more than 60% by weight or more than 80% (w/w) of said arabinoxylo-oligosaccharides.

Pharmaceutical compositions for the oral administration of the said arabinoxylo-oligosaccharides can take the form of solid formulations such as, for example, tablets or capsules prepared by conventional means with one or more pharmaceutically acceptable excipients such as, but not limited to, binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulfate). The tablets can be coated by methods well known in the art. Liquid formulations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be presented as a dry product, such as powders for dispersion or dissolution into water and/or other suitable liquid vehicles before use. Such liquid formulations can be prepared by conventional means with one or more pharmaceutically acceptable additives such as, but not limited to, suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); aqueous or non-aqueous vehicles (e.g. almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g. methyl- or propyl-p-hydroxybenzoates or sorbic

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acid). These formulations can also contain buffer salts, flavouring agents, colouring agents and/or sweetening agents, as appropriate.

In a particular embodiment of this invention, said medicament may be formulated as a powder that can be admixed with a food or drink product, wherein said powder comprises between 5 and 80% (w/w), more preferably between 10 and 60% (w/w), for instance between 30 and 50% (w/w) of said arabinoxylo-oligosaccharides. Said powder preferably further comprises a flowing agent, such as silica and optionally a filler. In another particular embodiment said medicament may be formulated as a syrup, preferably comprising between 8 and 50% (w/w), more preferably between 10 and 50% (w/w), for instance between 20 and 40% (w/w) of said arabinoxylo-oligosaccharides. Said syrup may further comprise an aqueous or non-aqueous vehicle, a suspending agent, an emulsifying agent, a preservative, a buffer salt, a flavouring agent, a colouring agent and/or a sweetening agent.

The pharmaceutical formulation of this invention may also take the form of, for example, a chewing gum, a confectionery, a gel, and the like. Next to arabinoxylo-oligosaccharides, a chewing gum composition of the present invention may include a gum base and one or more of the other typical chewing gum composition components such as sweeteners, softeners, flavouring agents and the like. The chewing gum compositions of the present invention, may be coated or uncoated and may be in the form of slabs, sticks, pellets, balls amongst other forms. In a further aspect of the present invention, the chewing gum compositions may include an insoluble gum base generally comprising one or more elastomers, plasticizers, waxes, fats, oils, emulsifiers, fillers and/or texturizers. Other ingredients used in chewing gum compositions may further include both natural and artificial, sugar-based and sugarless sweeteners. Sugarless sweeteners include, but are not limited to, sugar alcohols such as sorbitol, mannitol, xylitol, hydrogenated starch hydrolysates, maltitol and the like. High intensity sweeteners such as sucralose, aspartame, neotame, salts of acesulfame and the like may also be present. Flavouring agents for use in chewing gum compositions are well known in the art and include citrus oils, peppermint oil, spearmint oil, oil of wintergreen, menthol and the like. Softeners may be present to modify the texture of the chewing gum composition. Other materials which may be present in the gum composition of the present invention include antioxidants (e.g. butylated hydroxyanisole, butylated hydroxytoluene, β -carotenes, tocopherols, colorants, flavorants and the like.

The term "confectionery" as used herein includes, but is not limited to, nougats, candies, panning goods, gel confections, fondants, lozenges, hard boiled candies, mints, troches, pastilles, microcapsules, and other solid forms including freeze dried forms (cakes, wafers, and tablets) and fast dissolving solid forms including compressed tablets and water soluble thin films. Lozenges may include discoid shaped solids comprising said arabinoxylo-oligosaccharides in a flavoured base. The base may be a hard sugar candy, glycerinated gelatin, or a combination of sugar with sufficient mucilage to provide a stable form at room temperature. Lozenge compositions (compressed tablet type) typically include one or more fillers (compressible sugar), flavouring agents and/or lubricants. Details regarding the preparation of confectionery compositions can be found in Skuse's Complete Confectioner (13th Edition) (1957) the content of which is incorporated herein by reference.

It is preferred that the said daily effective dose of the arabinoxylo-oligosaccharides is between 1 to 10, more preferably 1 to 7, most preferably 1 to 5, for instance 3 or 4,

tablets, capsules, chewing gums, or confectionery used as a pharmaceutical composition for the administration of said arabinoxylo-oligosaccharides.

A preparation comprising said arabinoxylo-oligosaccharides, which is in particular suitable for use in animal feed can be produced starting from the 'thin stillage' derived from the industrial ethanol production using cereals such as wheat, corn, barley or rye. When ethanol-containing fermented mash from cereals, produced by yeast-mediated fermentation after mashing and enzymatic liquefaction and saccharification of the starch, is distilled to obtain ethanol, a slurry comprising an aqueous phase and solids is obtained, which is typically referred to as 'whole stillage'. The whole stillage can be separated through physical separation, such as filtration, centrifugation or decantation, in a liquid aqueous fraction called 'thin stillage' and a solid-enriched fraction called 'wet distilled grains'. On dry matter basis, the thin stillage from cereal fermentation contains between 20% and 40% (w/w) of said arabinoxylo-oligosaccharides. This product can be dried by a sequence of water evaporation and drying steps, either with or without mixing with a carrier product suitable for nutritional use, such as cereal bran, to facilitate the drying process. The ratio of the thin stillage dry matter to the dry matter of the carrier product can vary between 1:0 and 1:4 (w/w on dry matter basis). The arabinoxylo-oligosaccharides content of the final dry product depends on the ratio of thin stillage over the drying carrier, and preferably contains between 8 and 40% (w/w), more preferably between 15 and 40% (w/w), most preferably between 20 and 40% (w/w), for instance between 25 and 35% of said arabinoxylo-oligosaccharides. Preferably, such arabinoxylo-oligosaccharides-containing preparations are added to the feed in amounts allowing to supplement the feed with between 1 and 50 g, more preferably between 1 and 10 g, most preferably between 1 and 5 g of said arabinoxylo-oligosaccharides. For instance, when a feed is supplemented with such thin stillage derived arabinoxylo-oligosaccharides-containing preparation comprising 20% of arabinoxylo-oligosaccharides said preparation is preferably added to the feed in an amount varying between 5 and 250 g, more preferably between 5 and 50 g, most preferably between 5 and 25 g per kg of feed. Therefore, in a fourth object the present invention provides a method for the production of a preparation between 8 and 40% of arabinoxylo-oligosaccharide. This method comprises the separation of the aqueous phase (thin stillage) from a mash of yeast fermented cereals after distillation of the ethanol from said mash (whole stillage) and subsequently drying said isolated thin stillage.

Example 1

Effect of Arabinoxylo-Oligosaccharides (AXOS) on Infection of Chickens by *Salmonella*

Materials and Methods

AXOS Preparations.

AXOS-9-0.25 was prepared from wheat bran by extraction with a glycosyl hydrolase family (GHF) 10 endoxylanase essentially as previously described (Swennen et al. cited supra), except that the step of protease treatment of the bran was omitted. This preparation has a content in polymeric xylose and arabinose of 61% on a dry matter basis, the average degree of polymerisation of AXOS is 9, and the arabinose to xylose ratio is 0.25. AXOS-3-0.25 was prepared by enzymatic extraction from wheat bran as previously described (Swennen et al. cited supra), except that the xylanase treatment was performed for 10 hours at 50° C. with a GHF 11 xylanase (Grindamyl H640, commercially available from

Danisco, Denmark) at 1.2 units per g destarched and deproteinised wheat bran, and for another 10 hours at 50° C. after addition of a GHF 10 xylanase (Shearzyme 500L, commercially available from Novozymes, Denmark) at 21 units per g destarched and deproteinised wheat bran. This preparation has a content in polymeric xylose and arabinose of 81% on a dry matter basis, the average degree of polymerisation of AXOS is 3, and the arabinose-to-xylose ratio is 0.25.

Feed Composition.

The chicks were fed a single wheat-corn-soybean diet throughout the experiment. Four different diets were prepared. The control ration contained no added AXOS and had the composition shown in Table 1. The 0.2% AXOS-3-0.25 diet consisted of the control ration with addition of 0.25% (w/w) AXOS-3-0.25, which corresponds to 0.2% AXOS content after correction for purity. The 0.2% AXOS-9-0.25 diet consisted of the control ration with 0.33% (w/w) of added AXOS-9-0.25, corresponding to 0.2% AXOS content after correction for purity. The 0.4% AXOS-9-0.25 diet consisted of the control ration with 0.66% (w/w) of added AXOS-9-0.25, corresponding to 0.4% AXOS content after correction for purity.

Salmonella Strain.

Salmonella enterica ser. Enteritidis strain SE147, a well-characterized streptomycin-resistant strain isolated from a poultry farm was used for challenge of the chickens. The strain was grown for 6 hours in Luria-Bertoni medium (LB). The number of colony-forming units (cfu) per milliliter was determined by plating 10-fold dilutions of the bacterial suspension on brilliant green agar (BGA, commercially available from Oxoid, Basingstoke, United Kingdom) containing streptomycin (100 µg/ml), whereafter the plates were incubated aerobically for 20 hours at 37° C. The initial bacterial suspensions were kept at 4° C. during plate counting, and they were diluted in phosphate buffered saline (PBS) to the appropriate density prior to use in the in vivo trial.

In Vivo Trial.

One-day-old chicks of mixed sex (224 in total, Ross breed) were housed in a bio-contained poultry house. The birds were randomly divided in 4 groups of 56 each, and each group was reared in a 1 m² surface pen with a steel rod floor. Optimal house temperature was provided by a central water heating unit and infrared bulbs and a continuous light regime was used throughout the experiment. All birds received feed and water (1 drinker per pen) ad libitum. They were orally vaccinated against coccidiosis (Paracox-5, commercially available from Schering-Plough Animal Health, Brussels, Belgium) at 3 days post-hatch. The birds were orally inoculated with 2.5×10⁹ cfu *Salmonella* per animal at 14 days post-hatch. At the day of *Salmonella* inoculation, cloacal swabs were taken from all animals. At days 1, 3, 7, 11, 15, 19, and 23 after inoculation, cloacal swabs were taken from all animals remaining in the pens. At days 1, 3, 7, 11, 15, 19, and 23 post inoculation, a total of 8 birds per treatment group were euthanized by intravenous T61 injection and samples of caecum and spleen were taken for bacteriological analysis. The experiment was performed under supervision of the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Bacteriological Analysis.

Cloacal swabs were directly inoculated on BGA plates containing 100 µg/ml streptomycin, which were incubated overnight at 37° C. Swab cultures were scored 0 for no colonies, 1 for 1 to 10 colonies, 2 for 11 to 100 colonies, 3 for 101 to 1000 colonies, or 4 for more than 1001 colonies of *S. enterica*. After plating, the swabs were enriched by immersing in buffered peptone water and incubating overnight at 37°

C. For those samples that scored negative by direct plating of the swabs, the overnight buffered peptone water cultures were further enriched by addition of 1 mL of this suspension to 9 mL brilliant green tetrathionate broth. After overnight incubation at 37° C., a drop of this suspension was plated on BGA containing 100 µg/ml streptomycin. Samples of caeca and spleen were homogenized in 10 volumes of buffered peptone water, and 10-fold dilutions were made in buffered peptone water. For each dilution 6×20 µL drops were inoculated on BGA containing 100 µg/ml streptomycin and after overnight incubation (37° C.) the number of colony-forming units per gram of tissue was determined after counting the bacterial colonies. For samples that were negative after titration, pre-enrichment was performed as described above for the cloacal swabs. Samples that were negative for *Salmonella* presence after titration but positive after *Salmonella* enrichment were presumed to contain 0.5×10^1 cfu/g. Samples that were negative after enrichment were presumed to have 0 cfu/g. The SPSS 9.0 software was used for statistical analysis. Datasets on *Salmonella* score or *Salmonella* counts were analysed by multivariate ANOVA with days after inoculation and treatment group as independent variables, using the Scheffe error protection for inter-variable comparisons. The nonparametric Kruskal-Wallis test was used to check for inter-treatment effects on *Salmonella* score or *Salmonella* counts at each time point, and in case inter-treatment effects were detected ($P < 0.05$), the nonparametric Mann-Whitney test was used to determine significant differences between the control and each of the treatment groups. Datasets on percentages of *Salmonella*-positive swabs were analysed by logistic regression with days after inoculation and treatment group as independent variables. The Cochran-Q test was used to check for inter-treatment effects on percentages of *Salmonella*-positive swabs at each time point, and in case inter-treatment effects were detected ($P < 0.05$), the McNemar change test was used to determine significant differences between the control and each of the treatment groups.

To assess the effect of AXOS on intestinal colonisation by *S. enterica* ser. Enteritidis, as a representative of a enteric pathogen, an in vivo infection trial was performed on chickens. Four different treatment groups were compared; the control group was fed a regular wheat-corn-soybean based diet, the second group was fed the control diet base with addition of the short chain AXOS-3-0.25 at 0.2%, and the third and fourth groups received the control diet base supplemented with the medium chain AXOS-9-0.25 at 0.2% and 0.4%, respectively. Inoculation with *Salmonella* was performed when the chicks were 14 days of age, and colonisation of the intestines by *Salmonella* was monitored by taking cloacal swabs from all living animals and by enumerating *Salmonella* in the caeca from a sample of euthanized animals. Cloacal swabs were taken from all animals at the day of inoculation. None of these swabs revealed the presence of *Salmonella*, indicating that there was no infection with *Salmonella* prior to the artificial inoculation.

During the period from day 1 to 11 days post infection (dpi), more than 80% of the cloacal swabs in the control group tested positive for *Salmonella* presence, while the percentage of positive swabs steadily declined in the 15-23 dpi period (FIG. 1A). A significant reduction compared to the control group was observed in the 0.4% AXOS-9-0.25 group at 1, 3 and 11 dpi, but not at the 7 dpi time point, and the rate of positive swabs further declined from 15 to 23 dpi as for the control group. A similar decrease in *Salmonella* shedding was observed for the 0.2% AXOS-9-0.25 group, with significant reductions at 3 and 11 dpi compared to the control group. In the AXOS-3-0.25 group, a significant reduction of positive

swabs was only noticed at 11 dpi. The presence of *Salmonella* in cloacal swabs was also determined after enrichment of the swabs. With this method, almost 100% of the control group tested positive for *Salmonella* presence in cloacal swabs in the 1-11 dpi period, whereas a significant reduction was seen at 1, 3, and 11 dpi for the 0.4% AXOS-9-0.25 group (data not shown). Data on the *Salmonella* score of the cloacal swabs, reflecting the number of *Salmonella* cfu per swab, are presented in FIG. 2. Highest scores were noted for the control group in the 1-11 dpi period, followed by a decline in the period from 15 to 23 dpi. In the 1-11 dpi period, the *Salmonella* score of the cloacal swabs from the 0.4% AXOS-9-0.25 group reached a peak at 7 dpi at the same level as the control group, yet at all other time points (1, 3 and 11 dpi) the scores were significantly lower than those of the control group. The scores of the 0.2% AXOS-9-0.25 group were intermediate between those of the 0.4% AXOS-9-0.25 and control groups, whereas the 0.2% AXOS-3-0.25 group only showed a significant reduction at 11 dpi.

Measurement of *Salmonella* colonisation of the caecum yielded similar results as provided by the cloacal swab scores (FIG. 3). When analysed over all timepoints by multivariate ANOVA analysis, the reduction in caecal *Salmonella* counts compared to the control group was significant only for the 0.4% AXOS-9-0.25 group ($P < 0.001$). The mean log *Salmonella* colony-forming units per g caecal content of the 0.4% AXOS-9-0.25 group was consistently lower relative to the control group throughout the 1-11 dpi period, whereafter the counts dropped to similar low levels for both treatments. At 7 dpi, the apparent peak of infection in the control group, the *Salmonella* counts in the caeca of the 0.4% AXOS-9-0.25 group were 2 log units lower relative to the control group ($P < 0.01$). At this time point, a significant reduction of more than 1 log unit was observed in the 0.2% AXOS-9-0.25 ($P < 0.05$), whereas the reduction seen in the 0.2% AXOS-3-0.25 group was not significant.

The extent of *Salmonella* translocation to internal organs was assessed by measuring the *Salmonella* levels in spleen at different time points after infection (FIG. 4). When analysed over all time points by multivariate ANOVA, a significant reduction in *Salmonella* infection was detected for the 0.4% AXOS-9-0.25 group ($P < 0.001$) and the 0.2% AXOS-9-0.25 group ($P = 0.003$). At 7 dpi, the peak of colonization, the spleen tissues were less colonized by *Salmonella* in all AXOS treatment groups relative to the control group. The strongest relative decrease in *Salmonella* counts was observed at 7 dpi for the 0.4% AXOS-9-0.25 group, which showed a reduction by more than 2 log units ($P < 0.01$), followed by a reduction of about 2 log units for the 0.2% AXOS-9-0.25 group ($P < 0.01$), while a trend for reduced infection was seen for the 0.2% AXOS-3-0.25 group ($P = 0.1$).

In conclusion, dietary addition of AXOS helps to protect animals from intestinal colonisation of an orally applied bacterial pathogen, as well as systemic translocation of the pathogen to organs. The protection is dose-dependent, since the level of protection was, for all examined tissues, higher in the animals receiving 0.4% dose than those receiving 0.2% of AXOS-9-0.25.

Example 2

Effect of AXOS on Infection of Rats by *Salmonella*

AXOS Preparation.

AXOS-5-0.20 was prepared from wheat bran by extraction with a glycosyl hydrolase family (GHF) 10 endoxylanase as previously described (Swennen et al. cited supra), except that

the step of protease treatment of the bran was omitted. This preparation had an average degree of polymerisation of 5 and an arabinose to xylose ratio of 0.20.

Study Design.

6-week-old male rats (Wistar) are purchased from Elevage Janvier (Le Genest-St-Isle, France) and randomly assigned to 3 groups of 8 rats each. The rats are housed in stainless steel wire-bottom cages in an environmentally controlled room (22° C.) with a 14-10 hours light-dark cycle. Rats are given free access to water and to pellets (10 mm) of the 'control' diet (Table 4) during 6 days. After 6 days of adaptation on the control diet, the rats are randomly assigned to one of 3 different treatment groups, and the groups are each given free access during 10 days to pellets (10 mm) of one of the 3 diets described in Table 4, respectively, a control diet, a 2.5% AXOS diet and a 5% AXOS diet.

Animals were weighed and feed intake is measured 3 times per week. After said 10 days, all animals are orally infected with a clinical isolate of *Salmonella enteridis*, as described by Bovee-Oudenhoven et al. in Gastroenterology (1997) 113: 550-7. The animals are infected in the morning by gastric gavage with 0.5 ml of saline containing 3% (wt/vol) sodium bicarbonate with 5×10^8 viable *S. Enteridis*. The day before infection and 1, 3, 6 and 9 days after *Salmonella* infection, fresh faecal samples are collected. The number of *Salmonella* in the faeces are quantified by plating on Modified Brilliant Green Agar as previously described (Giaffer et al., 1991). Throughout the infection experiment the rats of each experimental group are fed their respective experimental diet.

In a second infection experiment with rats the effect of administration of AXOS in the feed on translocation to the spleen at day 2 after infection is investigated. The experimental set-up is similar to that described above. At the section day the animals are sacrificed and the spleen is aseptically removed, weighed and homogenized in sterile saline (10-fold dilution) and plated on Brilliant Green Agar for counting as described in (Oudenhove et al., 1994). Viable *Salmonella* counts are expressed as the total log 10 colony-forming units in the spleen.

The study shows a reduction of the fecal *Salmonella* excretion in the groups fed an arabinoxylo-oligosaccharide-containing diet, illustrating a stimulating effect of the arabinoxylo-oligosaccharides on the colonization resistance against *Salmonella*. The study further provides an indication that the oral administration of arabinoxylo-oligosaccharides to rats reduces the translocation of *Salmonella* to internal organs and more particularly to the spleen.

Example 3

Effect of Daily Intake of Different Dosages of AXOS on Gastro-Intestinal Complaints in Healthy Human Volunteers

AXOS Preparation.

AXOS-5-0.20 was prepared from wheat bran by extraction with a glycosyl hydrolase family (GHF) 10 endoxylanase as previously described (Swennen et al. 2006), except that the step of protease treatment of the bran was omitted. This preparation had an average degree of polymerisation of 5 and an arabinose to xylose ratio of 0.20.

Subjects

Sixty healthy volunteers (men and women; age ranging between 18 and 80 years) are included in the study. None of the subjects had severe gastrointestinal complaints in the 3 months prior to the start of the study and had ever had abdominal surgery. They have been free of antibiotics or any other

medical treatment influencing gut transit or the intestinal flora for at least 3 months before the start of the study. During the study the volunteers are urged to have a regular eating pattern (3 meals per day) and the intake of fermented milk products containing living bacteria or the intake of compounds known to modulate the composition of the intestinal flora is forbidden.

Study Design.

The duration of the entire clinical trial is 19 weeks. The clinical trial starts with a run-in period of 1 week, followed by 4 administration periods of 3 weeks each, with a wash out period of 2 weeks between the administration periods. In the course of the study all volunteers receive three dosages of AXOS-5-0.20 (2.5 g/day, 5.0 g/day and 10 g/day) and a placebo. The volunteers are divided into four groups in a random manner, with the same number of persons in each group, following a double blind protocol. The various treatments are assigned to the groups according to a 4x4 'Latin Square design', allowing the order of the treatments to be different for every group. The study design is schematically presented in FIG. 5.

During the administration periods, the volunteers are asked to consume 20 ml of a specific type of syrup at breakfast and in some administration periods an additional 20 ml at diner, this according to the intake scheme specified in Table 2. The compositions of the syrups used in the study is given in Table 3.

During the one-week run-in period and the last week of each intake period (AXOS or placebo), the volunteers are asked to fill in a questionnaire on a daily basis. The questions herein are designed to evaluate the occurrence and gravity of symptoms reflecting gastrointestinal complaints. The questions interrogate the volunteers on the occurrence of mild gastrointestinal discomfort, such as bloating or increased flatulence, as well as on more serious complaints, such as abdominal cramps, diarrhea and vomiting.

The outcome of the study indicates that the oral administration of arabinoxylo-oligosaccharides improves to overall gastrointestinal well being and lowers the occurrence of gastrointestinal discomfort and attenuates the gravity thereof. This suggests a better protection against gastrointestinal infection as a result of the intake of the arabinoxylo-oligosaccharides.

TABLE 1

		%
Feedstuffs		
Wheat		48.23
Yellow corn		10.00
Soybean meal-48		23.63
Heat full-fat soybean		8.50
Rendered animal fat		4.59
Soybean oil		1.25
Calcium carbonate		0.56
Calcium monohydrogen phosphate		1.27
Sodium chloride		0.29
Phytase (Ronozyme)		0.02
Sodium hydrogencarbonate		0.09
L-Lysine•HCl		0.28
DL-Methionine		0.23
L-Threonine		0.09
Vitamin & trace el. premix		1.00
sum		100.00
Composition		
Metabolisable energy for broilers, MJ/kg		12.40
Crude protein, %		20.75

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TABLE 1-continued

	%
Digestible lysine, %	1.13
Digestible sulphuric amino acids, %	0.79
Digestible threonine, %	0.73
Calcium, %	0.94
Available phosphorous, %	0.44
Cation-anion balance (Na+K—Cl), meq/kg	223.00
Linoleic acid, %	2.53

TABLE 2

Administration scheme for the AXOS and placebo syrups				
	Treatment			
	Placebo	2.5 g/dag AXOS	5 g/dag AXOS	10 g/dag AXOS
Break-fast	20 ml placebo syrup	20 ml AXOS syrup 2 (=2.5 g AXOS preparation)	20 ml AXOS syrup 1 (=5 g AXOS preparation)	20 ml AXOS syrup 1 (=5 g AXOS preparation)
Dinner	/	/	/	20 ml AXOS syrup 1 (=5 g AXOS preparation)

TABLE 3

Composition of syrups containing AXOS and placebo			
	Placebo syrup	AXOS syrup 1	AXOS syrup 2
AXOS-preparation (g/l)	0	250	125
Sucrose (g/l)	875	625	750
Carrageenan (g/l)	10	0	0
Colouring agent (ml/l)	3	3	3
Flavor (ml/l)	6	6	6
Citric acid (mg/l)	288	480	384
K-sorbate (mg/l)	200	200	200
Water (g)	365	365	365
TOTAL	1000 ml	1000 ml	1000 ml
TOTAL	1250 g	1250 g	1250 g

TABLE 4

Composition of the different rat diets (in g per 100 g).			
	control	AXOS 2.5%	AXOS 5%
Corn starch (pre-gelatinised)	73.50	71.00	68.50
AXOS preparation	—	2.50	5.00
Soy protein isolate	10.80	10.80	10.80
Wheat gluten	5.00	5.00	5.00
Soybean oil	3.50	3.50	3.50
L-Lysine	0.45	0.45	0.45
DL-Methionine	0.15	0.15	0.15
L-Cystine	0.08	0.08	0.08
L-Threonine	0.13	0.13	0.13
L-Tryptophan	0.07	0.07	0.07
Vitamin premix	1.00	1.00	1.00
Mineral/trace elem. premix	4.20	4.20	4.20

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TABLE 4-continued

Composition of the different rat diets (in g per 100 g).			
	control	AXOS 2.5%	AXOS 5%
Calcium carbonate	0.70	0.70	0.70
Cr ₂ O ₃	0.20	0.20	0.20
Choline chloride	0.20	0.20	0.20
Butylhydroxytoluol	0.02	0.02	0.02

The invention claimed is:

1. A method for reducing the infection of a non-human animal or a human being with *Salmonella* and reducing the translocation of said *Salmonella* towards internal organs, wherein said method comprises enterally administering a composition comprising an effective amount of an active ingredient, wherein said active ingredient consists of arabinoxylo-oligosaccharides having an average degree of polymerization between 3 and 50 and an average degree of substitution between 0.15 and 0.35.

2. The method according to claim 1, wherein said arabinoxylo-oligosaccharides are administered to said non-human animal through an animal feed.

3. The method according to claim 2, wherein the animal feed is supplemented with between 1 and 50 g of arabinoxylo-oligosaccharides per kg of feed.

4. The method according to claim 3, wherein the animal feed is supplemented with between 1 and 10 g of arabinoxylo-oligosaccharides per kg of feed.

5. The method according to claim 2, wherein said non-human animal is poultry or a livestock animal.

6. The method according to claim 2, wherein said non-human animal is a pet animal.

7. The method according to claim 6, wherein said arabinoxylo-oligosaccharides are administered to the pet animal by feeding the animal a pet food comprising between 0.25 and 5 g of arabinoxylo-oligosaccharides per daily serving of said pet food.

8. The method according to claim 6, wherein said arabinoxylo-oligosaccharides are administered to the pet animal by feeding the animal a pet treat food comprising between 0.25 and 5 g of arabinoxylo-oligosaccharides per serving of said pet treat food.

9. The method according to claim 6, wherein said pet is a dog or a cat.

10. The method according to claim 1, wherein said arabinoxylo-oligosaccharides are administered to said human being at a daily dose between 0.25 g and 10 g of arabinoxylo-oligosaccharides.

11. The method according to claim 10, wherein said arabinoxylo-oligosaccharides are administered at a daily dose between 0.5 g and 5 g of arabinoxylo-oligosaccharides.

12. The method according to claim 10, wherein said arabinoxylo-oligosaccharides are administered in a food product or non-alcoholic beverage.

13. The method according to claim 12, wherein said food product or non-alcoholic beverage is a cereal-based product comprising at least 25% of cereals or cereal-derived material.

14. The method according to claim 10, wherein said arabinoxylo-oligosaccharides are administered in a food supplement.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 9,061,046 B2
APPLICATION NO. : 12/680435
DATED : June 23, 2015
INVENTOR(S) : Willem Broekaert et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

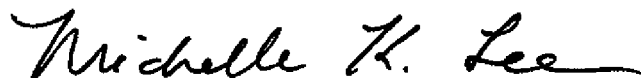
Column 1, line 62, replace “ \square -L-arabino-furanosyl” with -- α -L-arabino-furanosyl--.

In the Claims

Column 14, Claim 5, Line 29, replace “lifestock” with --livestock--;

Claim 10, Line 45, replace “ad ministered” with --administered--.

Signed and Sealed this
Seventeenth Day of November, 2015



Michelle K. Lee
Director of the United States Patent and Trademark Office